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Correlated with Melanocyte Migration and Diversification

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Melanocytes (Mc) and their progenitors melanoblasts (Mb) are derived from the neural crest and migrate along the dorsolateral pathway to colonize the dermis, the epidermis, and finally the hair matrix. To examine the involvement of cadherins in the migration of Mc lineage cells, we combined flow cytometric analysis of dissociated live cells with immunohistochemical staining of tissue sections to quantify the level of cadherin expression on the surface of Mb/Mc. At 11.5 days postcoitum, Mb are in the dermis and are E-cadherin$^-$P-cadherin$^-$ (E-cad$^-$P-cad$^-$). During the next 48 h, a 200-fold increase of E-cadherin expression is induced on the surface of Mb prior to their entry into the epidermis, thereby forming a homogeneous E-cad^{high}P-cad$^{-/low}$ population. The cadherin expression pattern then diversifies, giving rise to three populations, an E-cad$^-$P-cad$^-$ dermal population, E-cad^{high}P-cad^{low} epidermal population, and E-cad$^-$P-cad$^{med\text{-}high}$ follicular population. In all three populations, the patterns of expression are region-specific, being identical with those of surrounding cells such as keratinocytes and fibroblasts, and are preserved before and after pigmentation. While most of the epidermal Mb/Mc disappear after the neonatal stage in normal mice, forced expression of steel factor in the epidermis of transgenic mice promotes survival of epidermal Mb/Mc, maintaining epidermal-type cadherin expression pattern (E-cad^{high}P-cad^{low}) throughout the postnatal life. These findings indicate the involvement of extrinsic cues in coordinating the cadherin expression pattern of Mb/Mc and suggest a role for E- and P-cadherins in guiding Mc progenitors to their final destinations.

Key Words: **melanocyte; melanoblast; cadherins; E-cadherin; P-cadherin; expression; migration; diversification; colonization; quantitative; qualitative; microenvironment; steel factor (SLF); flow cytometry; epidermis; dermis; hair follicles; hair matrix.**

INTRODUCTION

In the mouse embryo, melanoblasts (Mb) are derived from the neural crest and migrate along the dorsolateral pathway between the dematome and the epidermis toward the ventral midline, dispersing over the dermis. At 12.5–13.5 days postcoitum (dpc) most Mb in the dermis enter the epidermis synchronously (Kunisada *et al.*, 1996; Mayer, 1973) where they proliferate extensively (Yoshida *et al.*, 1996). From 15.0 dpc, a subpopulation of epidermal Mb moves toward developing hair germs to localize in the hair matrix. While most Mb/melanocytes (Mc) eventually disappear from the epidermis during postnatal life (Hirobe, 1984), dermal Mc remain abundant in the dermis of hairless areas such as the ear and tail (Silvers, 1979). The mechanisms regulating such a specific migration and localization of the Mc lineage remain unknown (the process of Mc differentiation is depicted in Fig. 9).

The microenvironment surrounding Mb changes sequentially during Mc development, from the dermal mesen-

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chyme in the dorsolateral path, to the epidermis, and finally to the hair matrix. For Mb to migrate through such changing surroundings appropriately, the adhesive properties of Mb must be strictly regulated, both temporally and spatially. Integrins have been implicated in the migration of neural crest cells through the extracellular matrix (Bronner-Fraser, 1993, 1994; Perris, 1997). Regulation of Mb adhesion to surrounding cells may also be essential for guiding Mb to their destination, particularly during and after Mc entry into the epithelial layer where an epidermal architecture of keratinocytes is maintained by E- and P-cadherins (Hirai *et al.,* 1989). Interestingly, different cadherins are expressed along the Mb migration path. N-cadherin is expressed on the dermamyotome, cadherin-11 on the dermamyotome and a subpopulation of dermal mesenchymal cells, and E-cadherin on the epidermis while P-cadherin is expressed on the hair matrix and the epidermal basal layer (Hirai *et al.,* 1989; Inoue *et al.,* 1997; Takeichi, 1988). It is thus expected that the expression of cadherins on the surface of Mb is coordinated to be compatible with that of surrounding cells.

Cadherins are transmembrane glycoproteins connected to the actin cytoskeleton via cytoplasmic adaptor proteins andmediate homophilic adhesion between neighboring cells (Takeichi, 1988). Cell–cell adhesion via cadherins has been implicated in cell sorting behavior during morphogenesis (Takeichi, 1991). Moreover, it was recently implicated in cell migration also in *Drosophila* ovary (Niewiadomska, 1999). In respect to Mc, Tang *et al.* showed that cultured human Mc express E- and P-cadherins and that E-cadherin is primarily responsible for the adhesion of cultured Mc to keratinocytes *in vitro* (Tang *et al.,* 1994). Furthermore, dysfunction of E-cadherin has been implicated in melanoma progression and metastasis (Seline *et al.,* 1996). To our knowledge, however, none of the previous reports succeeded in determining cadherin expression during differentiation of Mc, probably due to technical difficulties in distinguishing Mb/Mc from surrounding cells.

We have established a method for analyzing and sorting freshly isolated Mb/Mc by flow cytometry (Kunisada *et al.,* 1996), which has enabled us to quantify the expression of various surface molecules in Mb/Mc. Using this method in combination with immunohistochemistry of tissue sections, we investigated the expression of cadherins by Mb/Mc at each developmental stage. The results show that an enormous change in the expression levels of E- and P-cadherins occurs during Mc development, eventually giving rise to three distinct subsets of Mc which are distinguished by differential expression levels of the two cadherins. The localization of the three Mc subsets correlated strongly with the expression patterns of the cadherins, which are identical with those of surrounding tissues. We suggest that such a coordinated cadherin expression by Mc lineage cells explains their specific migration and selective localization in the skin.

MATERIALS AND METHODS

Mice

C57BL/6 pregnant mice were purchased from Japan SLC (Shizuoka, Japan). Noon of the day the vaginal plug was detected was designated day 0.5 of gestation (0.5 dpc). The developmental stages of the embryos were judged by their morphological appearance as described in "The Atlas of Mouse Development" (Kaufman, 1992).

Antibodies

Anti-c-Kit monoclonal antibody (mAb) (ACK2) (Nishikawa, 1991), anti-E-cadherin mAb (ECCD-2) (Nose and Takeichi, 1986), and P-cadherin mAb (PCD-1) (Nose and Takeichi, 1986) were prepared and conjugated to either biotin or phycoerythrin (PE) (from Molecular Probes, Eugene, OR) as described previously (Nishikawa, 1991; Ogawa, 1991). Fluorescein isothiocyanate (FITC)-conjugated anti-CD45 mAb and biotinylated anti-αE integrin mAb were purchased from Pharmingen (San Diego, CA).

Rabbit anti-TRP2 antiserum, αPEP8 (Tsukamoto *et al.,* 1992), was a gift from V. J. Hearing. FITC-conjugated anti-rabbit polyclonal antibodies and biotinylated anti-rat polyclonal antibodies were purchased from Gibco (Grand Island, NY) and Biosource (Camarillo, CA), respectively. NeutraLight avidin-conjugated Texas red was from Molecular Probes.

Immunohistochemistry

Before fixation, decapitated embryos were kept in cold phosphate-buffered saline (PBS) containing 10 mM EDTA for 15 min to remove red blood cells from the tissues. Tissue fixation procedures were principally the same as described (Yoshida *et al.,* 1993), with microwave irradiation for prefixation proceeding for 20 s at 600 W in a microwave oven (Toshiba ER-365S, Tokyo Japan). Fixed specimens were embedded in polyester wax and sectioned at 6 μm as described (Kusakabe *et al.,* 1984). Sections were dewaxed and washed in PBS. Nonspecific staining was blocked by incubation with PBS containing 1% skim milk (Difco) for 20 min. For two-color immunostaining, sections were first incubated with primary antibodies overnight, washed three times, then incubated for 2 h with appropriate secondary fluorescence-labeled antibodies at room temperature. After three washes with PBS, sections were incubated with streptavidin-conjugated Texas red and washed again three times. Antibody binding was visualized using a fluorescence microscope (Axiophot, Zeiss, West Germany) combined with a color chilled 3CCD camera (C5810; Hamamatsu Japan) and superimposed using Adobe PhotoShop 4.0J.

Whole-mount immunohistochemistry was performed as previously described (Yoshida *et al.,* 1996) except that embryos were postfixed for 2–12 h in Dent's fixative, 93% methanol, and 7% dimethyl sulfoxide, after microwave fixation.

Preparation of Skin Cell Suspensions from Mouse Embryos

The procedure described previously (Kunisada *et al.,* 1996) was suboptimized to enhance the cell yield and maintain the antigenicity of cadherins. Skins of 20 to 70 embryos (depending on their stage) were removed en bloc from the dorsolateral trunk region with fine forceps in ice-cold PBS for each experiment.

The skins of 11- to 16.5-dpc embryos were incubated in Cell

Dissociation Buffer (Gibco) at 37°C for 5 min and gently dissociated by passing through 18- to 21-gauge needles. Then the samples were washed with Hanks' balanced salt solution (HBSS) including 1 mM $CaCl_2$, 0.005% DNase (Sigma), and 20% fetal calf serum (HyClone, Utah) (Wash Buffer) (Tang *et al.*, 1993). In the case of 13.5- to 16.5-dpc embryos, undissociated samples were centrifuged, washed with PBS, and incubated in approximately 10 times the volume of dispase solution (2.4 U/ml) (Boehringer Mannheim, Germany) in PBS (1 mM calcium) at 37°C for 7 min. After the samples had been gently dissociated by passing through an 18- to 21-gauge needle, digestion was stopped by adding Wash Buffer to the samples.

The samples of 17-dpc or older embryos and neonates were incubated with 0.25% (w/v) of type IV and type I collagenases in PBS at 37°C for 20–30 min. Epidermal sheets were peeled mechanically with fine forceps from digested dermal tissues using a dissection microscope. The peeled epidermal sheets and dermal cells were washed with 10 ml Wash Buffer and then with PBS. The samples were next incubated in cell dissociation buffer at 37°C for 5–10 min and dissociated well by passing through 18- to 21-gauge needles. The digestion was stopped by adding Wash Buffer to the samples.

Cell Surface Staining, Flow Cytometry, and Cell Sorting

Dissociated cells (1×10^6 cells) were incubated with 50 μl normal mouse serum for 20 min for blocking. Cells were then stained with PE-conjugated ACK2 and FITC-conjugated anti-CD45 and biotinylated anti-E- or -P-cadherin Ab for 20 min on ice. After being washed with HBSS with 1% bovine serum albumin (HBSS-BSA), cells were further stained with streptavidin conjugated to APC (Molecular Probes) for 15 min on ice. After being washed twice with HBSS-BSA, labeled cells were resuspended in HBSS-BSA containing propidium iodide (5 μg/ml; Sigma) to exclude dead cells and analyzed using a FACS Vantage (Becton–Dickinson, San Jose, CA). Cell sorting was performed as described previously (Ogawa *et al.*, 1991). Data were analyzed and printed out by using software CellQuest (Becton–Dickinson Immunocytometry Systems).

RT-PCR Analysis

Total RNA was isolated from 2×10^5 sorted cells, melanoblast cell line melb-a (Sviderskaya *et al.*, 1995), or 9.5-dpc whole embryos using Isogen (Nippon Gene, Japan). Oligo(dT)-primed cDNA was synthesized from each sample of total RNA using avian myeloblastosis virus reverse transcriptase as recommended by the manufacturer (Gibco). Using specific primers for each cadherin molecule, tyrosinase-related protein 2 (TRP2), leukocyte common antigen (CD45), and the housekeeping gene hypoxanthine phosphoribosyl transferase (HPRT), we performed PCR with the first strand of each cDNA. Primer pairs were as follows: E-cadherin, 5′ sequence (5′) 5-GCTGCCCCGAAAATGAAAAGGGTG-3 and 3′ sequence (3′) 5-TGTAGGGTAACTCTCTCGGTCCAG-3; P-cadherin, (5′) 5-AGGAGACGAAAGAGAGAGTGGGTG-3 and (3′) 5-GCCTCATACTTCTGCGGCTCAAAC-3; N-cadherin, (5′) 5-AGATTTCAAGGTGGACGAGGACGG-3 and (3′) 5-GGGTTCTCCACTTGATTGCCATTG-3; cadherin-6, (5′) 5-TTCAGACCAGGATAGAGGAGATGG-3 and (3′) 5-TCGGGGGTCTTAAACTGGTAGG-3; cadherin-11, (5′) 5-ATAGAAGAGTACACCGGGCCTGAC-3 and (3′) 5-AGCCTTCACCCTTCCTACTTCCTC-3; TRP2, (5′)-CAATTAAGGGTCAAGGGCTAGGGAG-3 and (3′) 5-TCTCGGCCATTGCTCACGGTCATC-3; CD45, (5′)-GAGCCAATCCATTCTGACATT-3 and (3′) 5-GTGGTCATTGATTGTCACAAC-3; HPRT, (5′)-GAGCTACTGTAATGATCAGTCAACG-3 and (3′) 5-GATTCAACTTGCGCTCATCTTAGGC-3. PCR conditions were optimized for each primer set to maintain amplification within the linear range.

Generation of SLF Transgenic Animals Driven by Cytokeratin 14 Promoter

Full-length mouse steel factor (SLF) cDNA that encodes both soluble and membrane-bound forms of SLF (Yasunaga *et al.*, 1995) was cloned into constructs containing the human cytokeratin 14 upstream region (Vassar *et al.*, 1989), rabbit β-globin intron, and poly(A) signal of human cytokeratin 14. Transgene excised from the plasmid sequence was injected into fertilized oocytes collected from $(C57BL \times SJL)F_1$ mice as described (Kimura *et al.*, 1995a). Integration of transgene was verified by PCR of genomic DNA with transgene-specific primers. The restriction map of the transgene was described previously (Kunisada *et al.*, 1998a).

RESULTS

Differential E-cadherin Expression on Dermal Melanocytes and Melanoblasts

To examine histologically whether Mc/Mb express E-cadherin *in vivo*, we first examined E-cadherin immunoreactivity in the hairless areas of the neonatal (2.5 days postpartum (dpp)) mouse skin where pigmented dermal Mc are abundant and easy to distinguish from surrounding cells (Fig. 1). E-cadherin was detected at cell–cell boundaries of epidermal keratinocytes as reported previously (Fujita *et al.*, 1992; Hardy and Vielkind, 1996b; Hirai *et al.*, 1989). Intriguingly, pigmented Mc in the dermis did not bind to anti-E-cadherin mAb (Fig. 1B, arrow). This result can be interpreted as either that E-cadherin is never expressed by Mb/Mc *in vivo* or that its expression is restricted to a certain developmental stage of the Mc lineage. Thus, we next examined E-cadherin expression in dermal Mb of an earlier stage.

We performed whole-mount immunohistochemistry with anti-E-cadherin mAb on 13.5-dpc skin. At this stage, Mb are still migrating in the dermis along the dorsolateral pathway. As shown in Fig. 2, a group of bipolar cells was detected in the dermis along both sides of the dorsolateral pathway (Figs. 2A and 2B). Their morphology and localization coincided with those of migrating Mb stained with anti-c-Kit mAb and anti-TRP2 antibodies at this stage (Kunisada *et al.*, 1996; Yoshida *et al.*, 1993, 1996). A higher magnification image of the same region showed that E-cadherin$^+$ cells in the dermis are distributed from just beneath the epidermal layer to the deep part of the dermis (Fig. 2B). Almost all E-cadherin$^+$ cells were scattered without forming clusters in the dermis despite their E-cadherin expression.

E-cadherin expression by Mc lineage cells was confirmed by double-immunofluorescent staining with anti-TRP2 antibodies (FITC) and anti-E-cadherin mAb (Texas red). As shown in

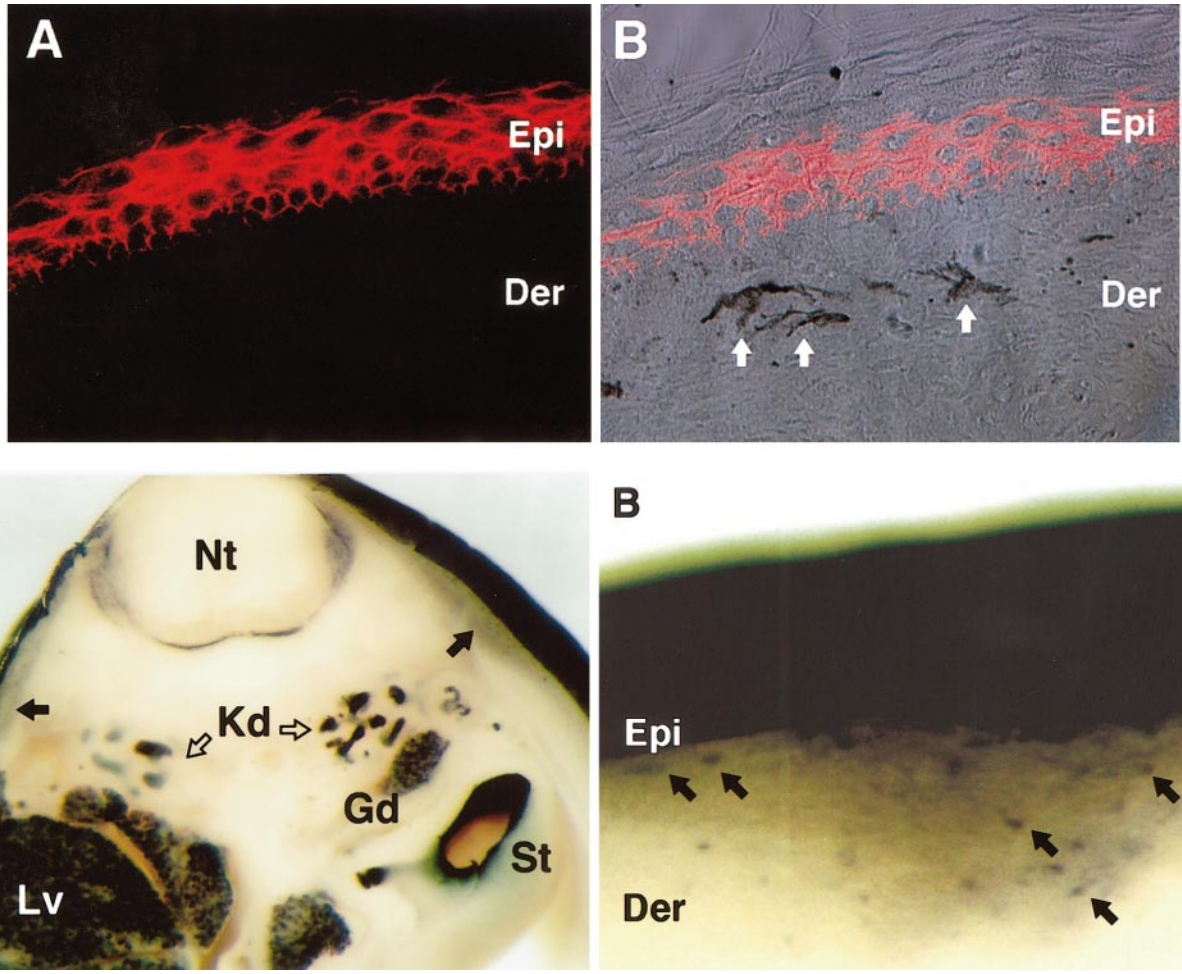


FIG. 1. Absence of E-cadherin immunoreactivity on dermal pigmented Mc. (A) Ear skin of 2.5-dpp mice was immunostained with anti-E-cadherin antibody (ECCD-2). (B) A phase micrograph of the same view was superimposed on (A). (A, B) E-cadherin immunoreactivity was detected at cell–cell boundaries of epidermal keratinocytes. Dermal pigmented Mc were negative for E-cadherin (closed arrows). Epi, epidermis; Der, dermis (original magnification 400).

FIG. 2. E-cadherin expression by dermal migrating Mb. (A) Embryos were processed for whole-mount ECCD-2 immunohistochemistry. Shown is a cross section of the trunk of a 13.5-dpc embryo. Positive signal was obtained in epithelial tissues, such as kidney (Kd), gonad (Gd), liver (Lv), and stomach (St). Groups of positive cells were detected on dermal mesenchyme along the dorsolateral pathway bilaterally. (B) A higher magnification of the cross section. Spindle- or bipolar-shaped E-cadherin-positive cells were found on dermal mesenchyme (Der) below the densely stained epidermis (Epi).

Figs. 3A–3C, $TRP2^+$ Mb in the dermis of 13.5-dpc embryo coexpressed E-cadherin. At 14.5 dpc, when Mb have completed entry into the epidermis, E-cadherin expression was also detected in those $TRP2^+$ Mb in the epidermis (Figs. 3J–3L). In the presumptive hairless areas in facial regions, some Mb remain in the dermis at 14.5 dpc (Figs. 3D–3I). Comparing with the E-cadherin expression level of keratinocytes as a reference, dermal Mb at 14.5 dpc appeared to express a lower level of E-cadherin than those at 13.5 dpc (Figs. 3A–3I). After 16.5 dpc, E-cadherin expression could not be detected histologically in Mb/Mc in the dermis (Figs. 6C and 6D). While these results suggest a marked variation of E-cadherin expression level during Mc development, fluorescent immunostaining is not sufficient for quantitative measurement of the expression level. Therefore, we employed the flow cytometric analysis which we had established previously for analyzing the expression of surface molecules on Mb/Mc dissociated from embryos (Kunisada *et al.*, 1996).

Quantitative Changes of E- and P-cadherin Expression during Melanocyte Differentiation

To enhance the cell yield during enzyme digestion, while preserving epitopes of cell-surface molecules, a previous

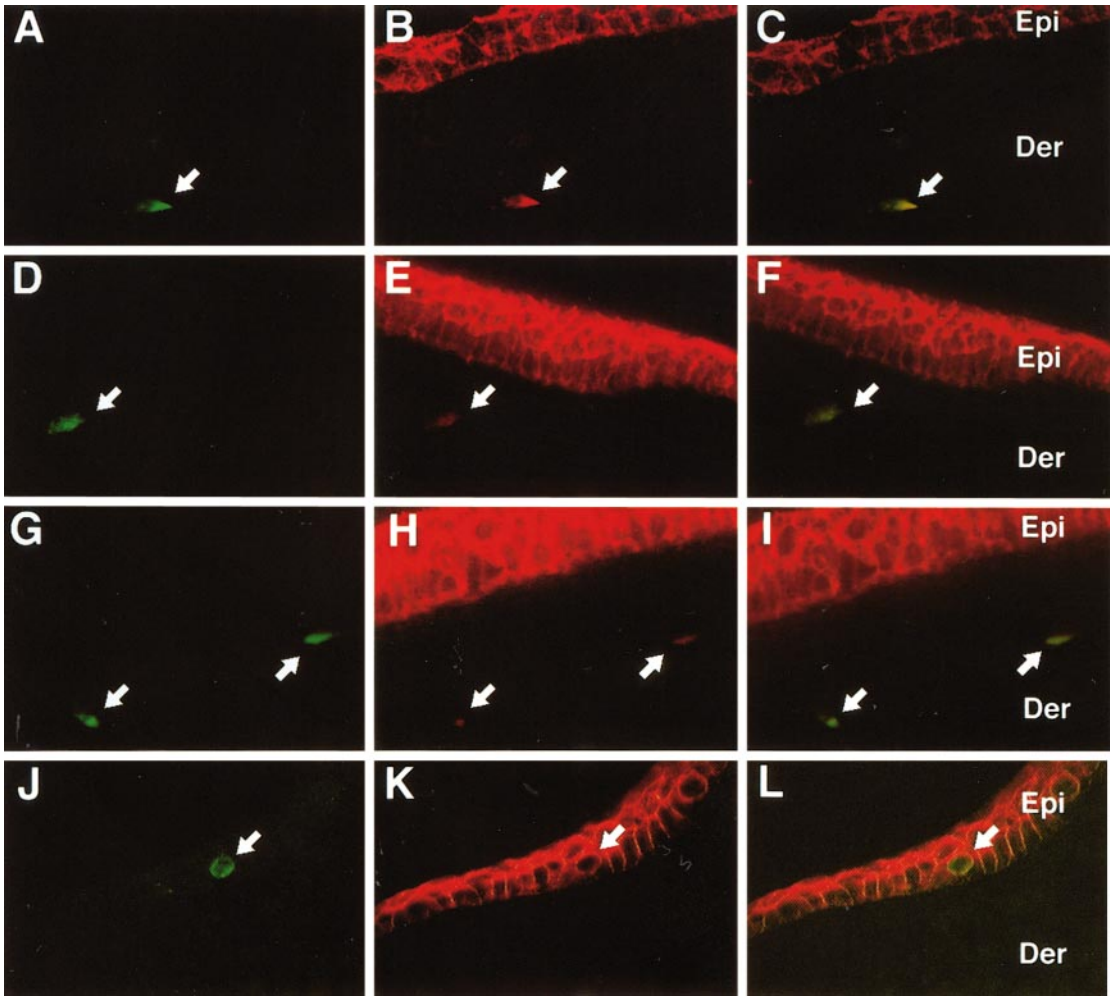


FIG. 3. E-cadherin immunoreactivity on dermal and epidermal Mb. (A–C) Transverse section of 13.5-dpc facial skin where entry of Mb into the epidermis is in progress. (D–L) Transverse section of 14.5-dpc facial skin where the entry is finished. (A, D, G, J) Staining of Mb with anti-TRP2 antibodies (green). (B, E, H, K) The same sections as in (A, D, G, J), respectively; immunostaining for E-cadherin (red). (C, F, I, L) Superimposed images of (A, D, G, J) and (B, E, H, K), respectively. Epi, epidermis; Der, dermis (original magnification 400).

method for cell dissociation was modified as described under Materials and Methods. Our previous studies showed that TRP2$^+$ Mb are represented by the population of c-Kit$^+$CD45$^-$ surface phenotype (Kunisada *et al.*, 1996). In order to confirm this for the cells prepared by the new method, we stained cells dissociated from the skin at 10.5 dpc to 0.5 dpp of age and analyzed them by flow cytometry. Though we could not identify c-Kit$^+$CD45$^-$ cells in the preparation from 10.5-dpc embryos, this population could be detected after 11.5 dpc (data not shown). A representative flow cytometric profile of cells prepared from 13.5-dpc embryos is presented in Fig. 5. The c-Kit$^+$CD45$^-$ and c-Kit$^+$CD45$^+$ factions were sorted from this preparation to 97.0% purity (Fig. 4) and used for either immunostaining by anti-TRP2 antibody or RT-PCR analysis of the various molecules indicated in Fig. 4B.

In agreement with our previous study (Kunisada *et al.*, 1996), 80–90% of the c-Kit$^+$CD45$^-$ population were TRP2$^+$, while none of the c-Kit$^+$CD45$^+$ cells were (data not shown), confirming that most, if not all, of the c-Kit$^+$CD45$^-$ cells are actually of the Mc lineage.

The expression of cadherins in the c-Kit$^+$CD45$^+$ and c-Kit$^+$CD45$^-$ fractions was analyzed by RT-PCR. In addition to E-cadherin, we analyzed the expression of P-cadherin, N-cadherin, cadherin-6, and cadherin-11, because those four have been shown to be expressed in the tissues along the migration route of Mb (Hatta *et al.*, 1991; Kimura *et al.*, 1995b; Inoue *et al.*, 1997). None of these cadherins were detected in the c-Kit$^+$CD45$^+$ fraction representing hematopoietic lineage cells. On the other hand, the c-Kit$^+$CD45$^-$ fraction expressed both E- and P-cadherins but not N-cadherin, cadherin-6, nor cadherin-11. Interest-

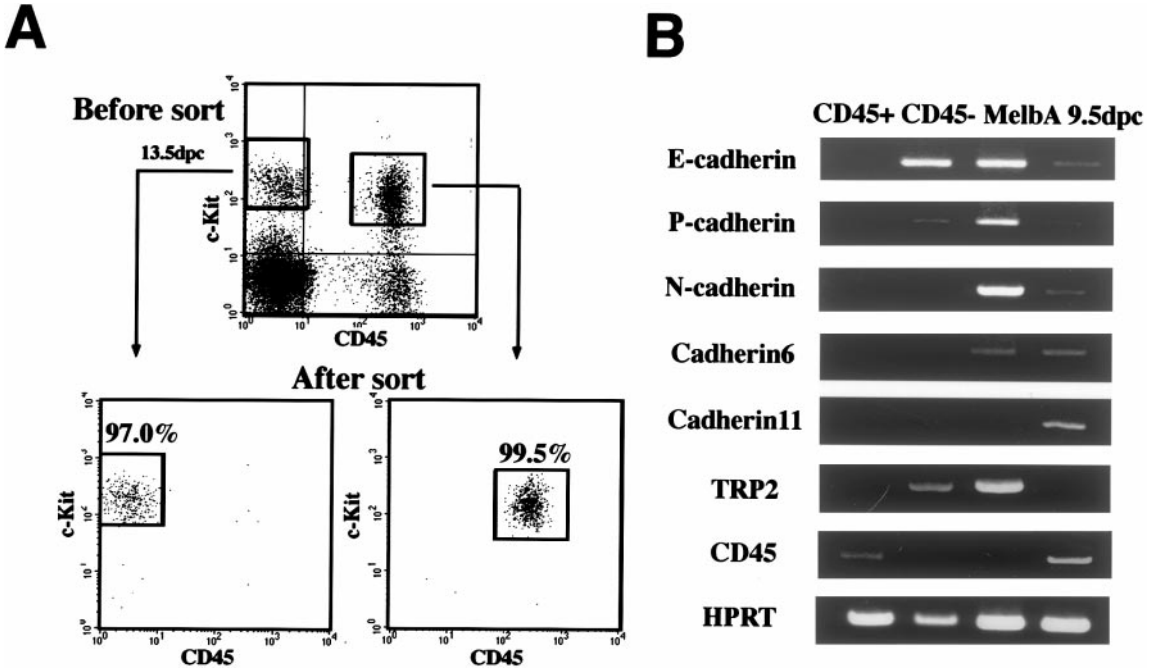


FIG. 4. Isolation of Mb/Mc by flow cytometry and analysis of cadherin mRNA expression in sorted cells. (A) Flow cytometric analysis of c-Kit and CD45 expression on cells prepared from 13.5-dpc fetal skin. Dissociated cells were incubated with anti-CD45 and anti-c-Kit mAbs. c-Kit$^+$CD45$^-$ cells and c-Kit$^+$CD45$^+$ cells were gated and sorted. The purity of sorted cells is also presented. (B) Reverse transcriptase-PCR analysis of total cellular RNA from c-Kit$^+$CD45$^-$ sorted cells (CD45$^-$), c-Kit$^+$CD45$^+$ sorted cells (CD45$^+$), melanoblast cell line (MelbA) and 9.5-dpc whole embryo (9.5 dpc). Data from three representative experiments are shown. HPRT mRNA served as a control in each experiment.

ingly, the melb-a Mb cell line expressed N-cadherin and cadherin-6 mRNA as well as E- and P-cadherin mRNA. Thus, this cell line does not represent embryonic Mb in terms of the cadherin expression pattern.

We next attempted to quantify the expression level of E- and P-cadherins during Mc differentiation. Dissociated cells were three-color stained and E-cadherin expression in the c-Kit$^+$CD45$^-$ fraction was measured. As summarized in Fig. 5A, an enormous change in the E-cadherin expression level was observed during Mb development. At 11.5 dpc, most Mb were E-cadherin$^-$. Within the next 48 h, a striking increase in the E-cadherin expression level of over 200-fold was induced in all Mb, eventually giving rise to a homogeneous E-cadherin^{high} (E-cad^{high})[2] population. After reaching this peak level, the expression was downregulated differentially, giving rise to diverse populations which express E-cadherin to varying extents (Fig. 5A).

While only a few c-Kit$^+$CD45$^-$ cells were P-cad$^+$ at 11.5 dpc, the expression level increased gradually from 11.5 to 15.5 dpc. Interestingly, a small population of P-cad$^{med\text{-}high}$ cells appeared at around birth (Fig. 5B). Comparing with E-cadherin expression, we could not detect a sharp peak in P-cadherin expression level in the c-Kit$^+$CD45$^-$ cells. We arbitrarily separated the small P-cad$^{med\text{-}high}$ small population (10^2–10^3 in histogram) which appeared during 15.5 dpc and 0.5 dpp from the major P-cad$^-$ population.

These results show that cadherin expression by Mb is regulated quantitatively and qualitatively during Mb migration and suggest that coordinated expression of E- and P-cadherins on the Mb cell surface is involved in heterotypic adhesion with keratinocytes. We also examined the expression of αE-integrin on developing Mb, because $\alpha E\beta 7$ was known to be involved in lymphocyte interaction with E-cad$^+$ epithelial cells (Cepek *et al.*, 1994). Expression of αE-integrin was not detected in the c-Kit$^+$CD45$^-$ population throughout the developmental process (data not shown).

Three Melanocyte Subsets Which Differ in E- and P-cadherin Expression

We did not expect such a diverse set of Mb to be generated during embryogenesis. On flow cytometry, however, all information about the spatial position of the cells was inevitably lost. Thus, we explored a possibility that the diversity in cadherin expression patterns correlates with

[2] Abbreviations used: med, intermediate; E-cad, E-cadherin; P-cad, P-cadherin.

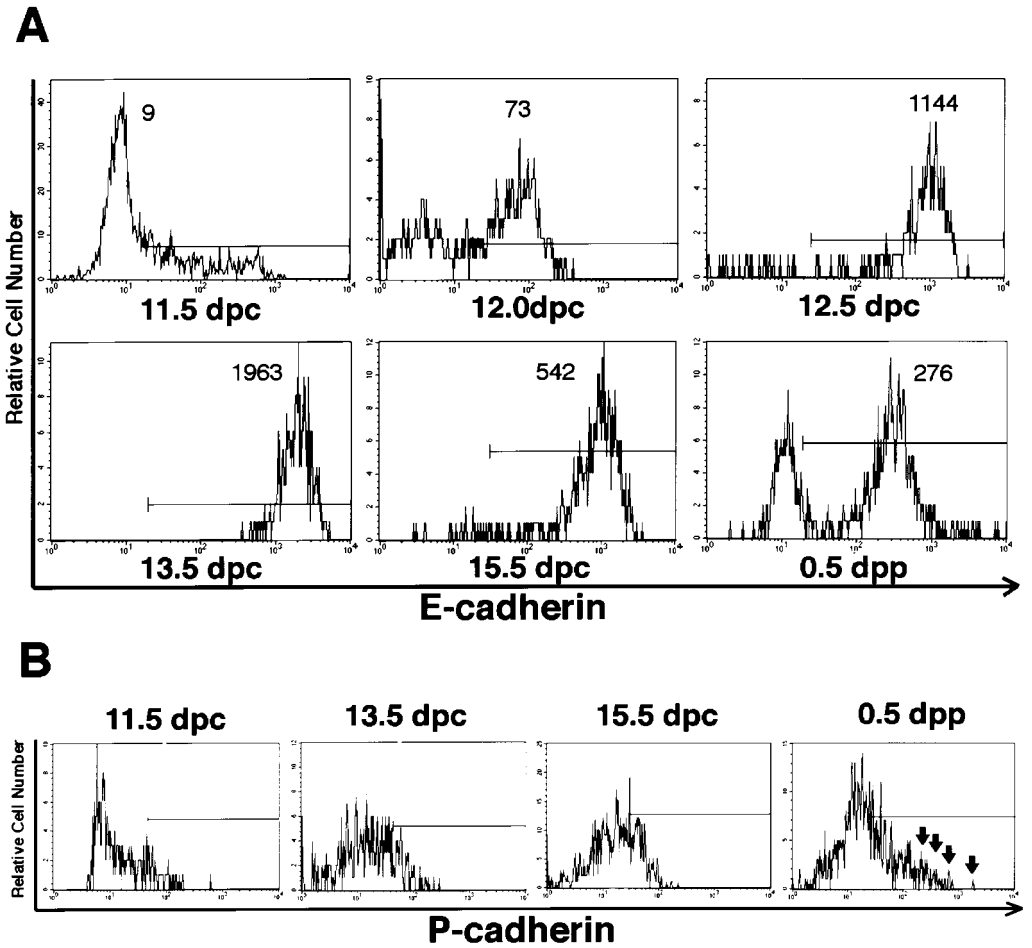
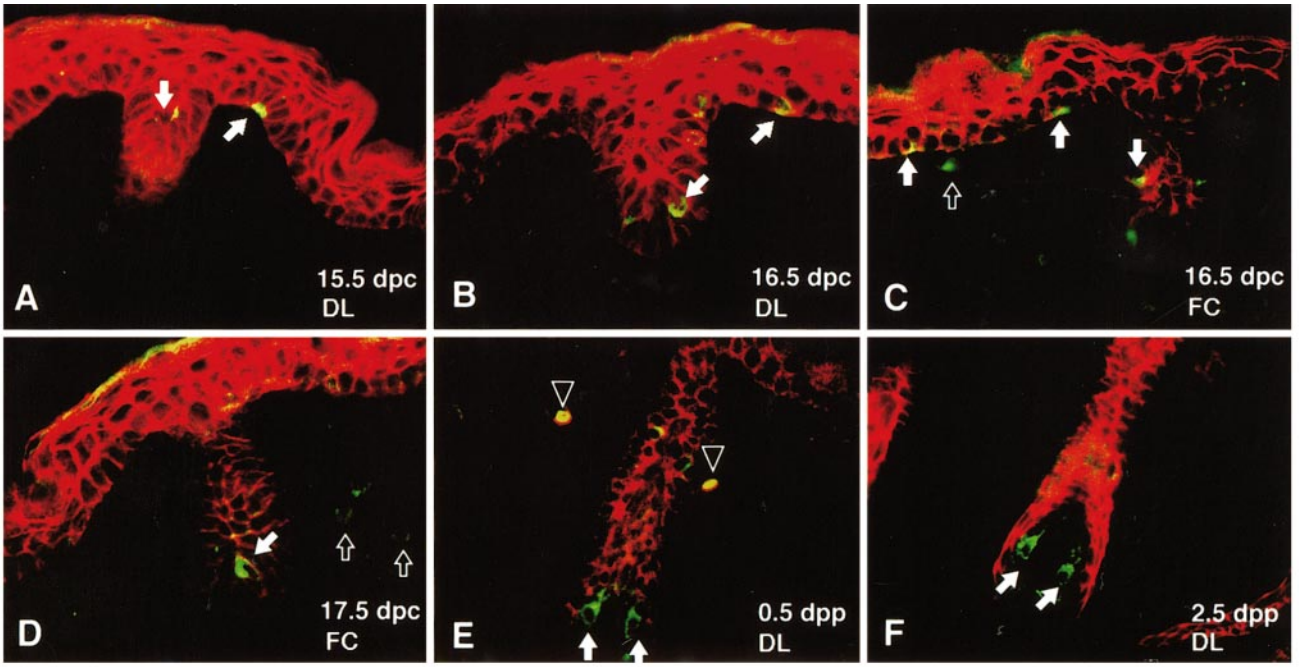


FIG. 5. Quantitative analysis of E-cadherin and P-cadherin expression on Mb/Mc. (A, B) Cell suspensions prepared at each developmental stage were stained with anti-CD45, anti-c-Kit, and anti-E- or -P-cadherin mAbs. E- and P-cadherin expression was analyzed in a c-Kit$^+$CD45$^-$ gated population by flow cytometry. Gated region in each histogram (horizontal line) was determined by isotype-matched control staining (anti-CD8 mAb). (A) E-cadherin expression markedly increased between 11.5 and 12.5 dpc. At 13.5 dpc, Mb show uniform high expression of E-cadherin. Between 15.5 and 0.5 dpp, the proportion of E-cadherin-negative cells increased, while the major population show E-cad^{high}. The peak value of each histogram was shown near the peak. (B) P-cadherin expression was also examined at different stages. The expression level increased gradually after 11.5 dpc. A P-cad^{high} population appeared at later stages (arrows).

the localization of Mb/Mc. For this purpose, we examined skin sections of 15.5 dpc–0.5 dpp, when we could detect the diversity of Mb/Mc by flow cytometry, with anti-E-cadherin or anti-P-cadherin mAbs in combination with TRP staining for identifying Mb/Mc.

As shown in Figs. 6 and 7, TRP2$^+$ cells were found in different locations, including the dermis, epidermis, and developing hair follicles after initiation of hair budding. All dermal TRP2$^+$ cells are negative for E-cadherin after 16.5 dpc, while those in the epidermis are E-cad$^+$ (Fig. 6). Unlike the TRP2$^+$ cells remaining in the epidermis, the E-cadherin expression level in those incorporated into the hair follicles decreases, eventually giving rise to E-cad$^-$ cells in the hair matrix. Concomitant with the downregulation of E-cadherin expression, P-cadherin expression is upregulated in TRP2$^+$ cells in the hair follicle, though in the fully mature follicle TRP2$^+$ cells again downregulate P-cadherin expression (Fig. 7). P-cadherin expression could not be detected on TRP2$^+$ cells in the dermis at any stage. The changes of E- and P-cadherin expression patterns in surrounding keratinocytes of the epidermis and hair follicles were evaluated in the same sections. Our results corroborate well with previous reports (Hirai *et al.*, 1989; Hardy and Vielkind, 1996a), in that E-cadherin expression was completely lost in the keratinocytes of hair matrix, while the P-cadherin expression level increased. Mb/Mc display the same patterns as the surrounding keratinocytes of the



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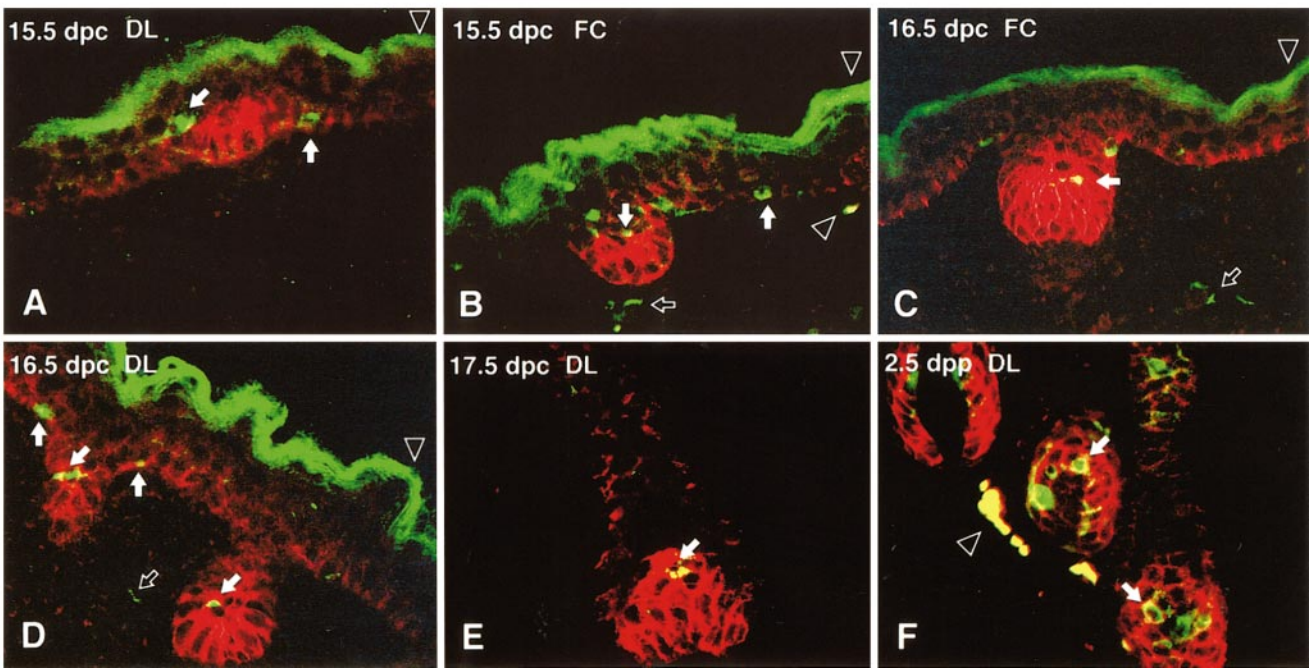

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FIG. 6. E-cadherin expression is downregulated on dermal and follicular Mb/Mc in late developmental stages. Staining for E-cadherin (red) and TRP2 (green) of transverse section of hairless facial skin (FC), where dermal Mb exist (C, D), and dorsolateral skin (DL), where dermal Mb are rarely found (A, B, E, F), at different developmental stages. (A, B, C) E-cadherin immunoreactivity was detected at cell–cell boundaries of epidermal cells but was reduced in the basal layer of hair buds. $TRP2^+$ cells in the epidermis and hair follicles are marked with closed arrows. $TRP2^+$ Mb in the epidermal basal layer were positive for E-cadherin. $TRP2^+$ cells were detected in the suprabasal layer of developing hair buds. In hairless facial skin (C, D), $TRP2^+$ Mb remained in the dermis and were negative for E-cadherin at 16.5 dpc and later (open arrows). (E) E-cadherin immunoreactivity is not detected on $TRP2^+$ cells close to the hair matrix. Open arrowhead, nonspecific staining of red blood cells. (F) $TRP2^+$ cells localized in the hair matrix are also negative for E-cadherin (original magnification 400).

FIG. 7. Follicular Mb/Mc upregulate P-cadherin expression during migration into the hair matrix. Staining for P-cadherin (red) and TRP2 (green) of transverse sections of hairless facial skin (FC), where dermal Mb exist (B, C), and dorsolateral skin (DL), where dermal Mb are rarely found (A, D, E, F), at different developmental stages. (A, B, C, D) P-cadherin immunoreactivity was detected at cell–cell boundaries

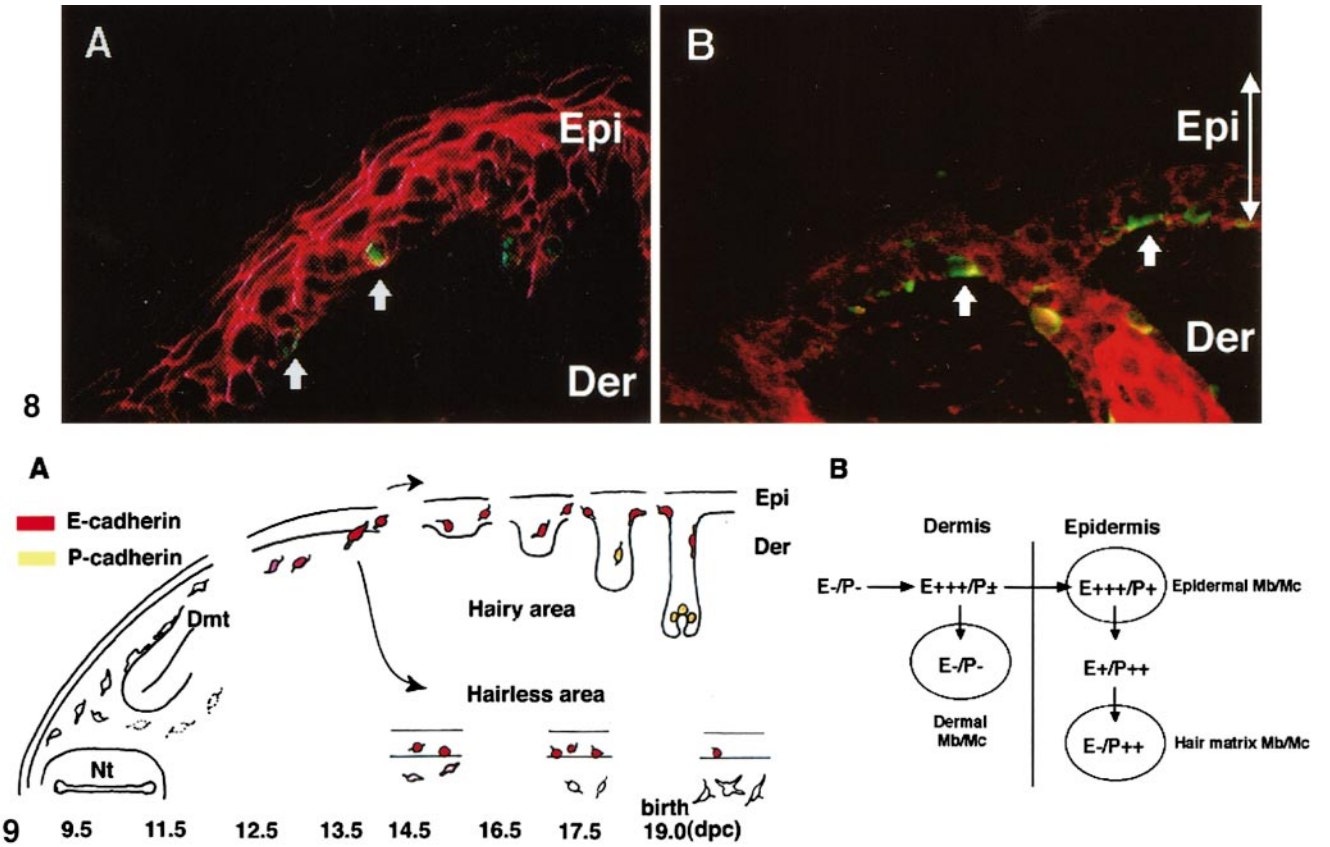

FIG. 8. Sustained E-cadherin expression on epidermal Mc surviving in the epidermis of SLF-transgenic mice of postnatal stage (10 dpp). (A) Staining for E-cadherin (red) and TRP2 (green) of the dorsal skin. (B) Staining for P-cadherin (red) and TRP2 (green) of transverse sections of dorsal skin. (A, B) Note the colocalization of E- or P-cadherin signal and TRP2 signal detected at the interface between epidermal Mc (arrows) and the basement membrane as well as between epidermal Mc and surrounding keratinocytes (original magnification 400).

FIG. 9. Summary scheme. (A) Switching on and off of cadherin expression during Mc development. Mb express neither E-cadherin nor P-cadherin at the early stage and upregulate the E-cadherin expression level markedly between 11.5 and 12.5 dpc before emigrating into the epidermis. Mb express high levels of E-cadherin homogeneously at 13.5 dpc. Mb remaining in the dermis downregulate E-cadherin expression, which is eventually lost on maturation. Migrating Mb within the hair buds downregulate E-cadherin while upregulating P-cadherin expression. Mc which reside in the hair matrix express high–intermediate levels of P-cadherin but not E-cadherin. (B) Cadherin expression associated with the diversification of Mc lineage. E and P represent E- and P-cadherin expression, respectively.

epidermis and hair follicles in terms of expression of E- and P-cadherin.

Collectively, immunohistological and flow cytometric results showed that the homogeneous E-cad^{high}P-cad$^{-/low}$ dermal Mb found in 13.5-dpc embryos (Fig. 5) develop into three distinct populations; E-cad^{-}P-cad^{-} dermal Mb/Mc, E-cad^{high}P-cad^{low} epidermal Mb/Mc, and E-cad^{-}P-cad$^{med\text{-}high}$ follicular Mb/Mc (summarized in Fig. 9B).

Evidence for Environment-Directed Regulation of the Cadherin Expression Pattern in Melanocyte Lineage Cells

The strong correlation between the cadherin expression pattern and localization of Mb/Mc suggests extrinsic rather than intrinsic regulation of the expression in Mb/Mc. If so, epidermal and dermal Mc should maintain E-cad^{high}P-cad^{low}

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of the basal keratinocytes at low level and hair germ keratinocytes at high level. TRP2^{+} cells in the epidermis and hair follicles are marked with closed arrows. (A) TRP2^{+} P-cad$^{-/low}$ cells are adjacent to P-cadherin-expressing keratinocytes. (B) TRP2^{+} cells are entering the developing hair buds. (B, C) In hairless areas of facial skin, TRP2^{+} Mb remained abundant in the dermis and were negative for P-cadherin (open arrow). (C, D) TRP2^{+} P-cad^{+} cells are migrating along the suprabasal layer of developing hair germs. (E, F) Strong P-cadherin signal was detected on cell–cell boundaries of the hair matrix keratinocytes. (F) P-cadherin is expressed on the cell surface of TRP2^{+} cells settled in the hair matrix. Open arrowhead, nonspecific staining of the periderm (A, B, C, D) and red blood cells (B, F) by FITC- or Texas red-conjugated secondary Abs (original magnification 400).

and E-cad$^-$P-cad$^-$ phenotypes, respectively, throughout their life. As shown in Fig. 1, the dermal Mc present in hairless areas are indeed E-cad$^-$ even after they are fully pigmented. Whether this is also true for cadherin expression in epidermal Mc, however, has been difficult to test, because mouse epidermal Mb/Mc disappear from the epidermis between 4 and 10 dpp (Hirobe, 1984). To address this question, we took advantage of SLF-transgenic mice in which SLF was constitutively expressed by basal keratinocytes, thereby allowing epidermal Mb/Mc to survive even in the postnatal life (Kunisada *et al.*, 1998b). Interestingly, epidermal Mb/Mc surviving in the epidermis of transgenic mice show high levels of E-cadherin and low levels of P-cadherin expression, maintaining the epidermal-type cadherin expression pattern seen in fetal Mb of wild-type mice (Fig. 8). Furthermore, Mb/Mc display the same patterns as the surrounding keratinocytes of the epidermis in terms of expression of E- and P-cadherin. Pigmented Mc in the epidermis of the transgenic mice were also E-cad^{high}P-cad^{low}, indicating that this pattern correlates with the localization rather than the differentiation stage of Mb/Mc. These observations strongly suggest the involvement of an extrinsic cue in determining the cadherin expression pattern of Mb/Mc.

DISCUSSION

While the expression of E- and P-cadherins has been demonstrated in cultured Mc, the expression in normal Mb/Mc during embryogenesis has not. By flow cytometric analysis of the cells dissociated from mouse skin, we demonstrated that E- and P-cadherins are indeed expressed in developing Mb/Mc. Our *in vitro* adhesion assay for freshly isolated embryonic Mb and E-cad$^+$P-cad$^+$ keratinocytes showed that the two cadherins on the surface could mediate cadherin-dependent adhesion of Mb to the keratinocyte monolayer, which could be inhibited, though only partially, by blocking antibodies to either molecule (Nishimura *et al.*, unpublished data). Thus, two cadherins detected on the surface of embryonic Mb are functional in terms of their ability to mediate cell–cell adhesion as reported previously with cultured Mc (Tang *et al.*, 1994).

It should be emphasized that this is the first study to succeed in quantifying the expression level of cadherins during embryogenesis. Up- and downregulation of cadherins has been demonstrated in various processes of morphogenesis (Takeichi, 1991). To our knowledge, however, previous studies have provided only a rough estimation of the expression level by fluorescent immunohistochemistry. Flow cytometry of live cells enables us to compare the expression level of surface molecules on a quantitative basis at the single-cell level.

Surprisingly, the level of E-cadherin expression varied over 200-fold during the development of Mb (Fig. 5A). The most remarkable change occurs between 11.5 and 13.5 dpc. The expression level of E-cadherin peaked just before the entry into the epidermis. This time course of E-cadherin expression seems reasonable, as low E-cadherin expression may prevent self-aggregation and premature colonization into the epidermis during the migration of Mb in the dermis. It should be noted that lower level of E-cadherin is detectable in more than half of the Mb at 12.0 dpc, when most Mb remain in the dermis. This observation suggests that E-cadherin expression exceeding a threshold level is required for Mb adhesion with the epidermal layer. In fact, Steinberg *et al.* demonstrated *in vitro* that two cell types expressing an identical cadherin but at different levels segregate from one another, while those expressing at the same levels aggregate (Steinberg and Takeichi, 1994). Taken together, these observations imply that the cadherin expression level is an important factor in the invasion by heterotypic cells of the epithelial layer.

According to our previous study, the differentiation of Mc initially proceeds in a rather nonuniform fashion, faster in head and tail regions, slower in the trunk region (Yoshida *et al.*, 1996). Then, Mb differentiate synchronously between 12.5 and 13.5 dpc to colonize the epidermis within a short time window (Kunisada *et al.*, 1996; Yoshida *et al.*, 1996). It has been difficult to correlate this synchronization process to the phenotypic changes of Mb. We here showed that, in terms of the E-cadherin expression level, Mc lineage cells form a most uniform population at 13.5 dpc (Yoshida *et al.*, 1996), suggesting that regulation of the E-cadherin expression level on the surface of Mb is an important factor for the synchronous entry of Mb into the epidermis. Interestingly, our previous results showed that the injection of an antagonistic anti-c-Kit mAb at this timing resulted in mice which lack pigment cells over the entire coat, suggesting that the Mb in 13.5-dpc embryo are also homogeneous in terms of c-Kit dependency (Yoshida *et al.*, 1996). However, it should be emphasized that our study remains at population level rather than the fate analysis of each individual Mb. Thus, it is difficult to rule out the possibility that such a seemingly homogeneous Mb population has already diversified in terms of the future fate.

During the colonization by Mb of the basal region of the hair follicle, the hair matrix, a distinct change in cadherin expression patterns was observed in Mc lineage cells again. During the process, E-cadherin expression is downregulated on the one hand, and P-cadherin expression upregulated on the other, giving the E-cad$^-$P-cad$^{med\text{-}high}$ population. Interestingly, a similar change is observed during the development of hair follicles, in which epidermal keratinocytes give rise to hair matrix keratinocytes through hair germ keratinocytes (Hardy and Vielkind, 1996; Hirai *et al.*, 1989). This concordant expression of cadherins between Mb/Mc and surrounding cells may play a role in the correct positioning of Mb/Mc. Of note is that Mb dispersed in the epidermis undergo migration through hair germ keratinocytes to settle in the hair matrix. How Mb/Mc migrate through the organized tissue expressing the same set of cadherins is an interesting issue for future study. It was shown that both the oocyte and the follicle cells in *Drosophila* ovary express

DE-cadherin and the migration of these cells is regulated by changing the amount of DE-cadherin (Godt and Tepass, 1998; Niewiadomska, 1999). Such a striking resemblance between the Mc in hair follicles and the follicle cells in the *Drosophila* ovary tempts us to speculate that similar molecular mechanisms operate in the two systems. Since the expression of both E- and P-cadherins is differentially regulated in Mb/Mc, underlying mechanisms regulating Mc migration in the hair follicle would be more complicated.

Three distinct populations in the Mc lineage were identifiable in the neonatal stage Mb/Mc by flow cytometry in combination with immunohistochemistry: an E-cad$^-$P-cad$^-$ dermal population, E-cad^{high}P-cad^{low} epidermal population, and E-cad$^-$P-cad$^{med\text{-}high}$ follicular population. The E-cadherin expression level of epidermal Mc of 0.5 dpp is slightly (two- to threefold) lower than that of 13.5-dpc Mb. Thus, an E-cadherin expression level as high as that of 13.5-dpc Mb may not be required for maintaining Mc lineage cells in the epidermis. Interestingly, those three populations are found in three different regions of the skin: E-cad$^-$P-cad$^-$ in the dermis, E-cad^{high}P-cad^{low} in the epidermis, and E-cad$^-$P-cad$^{med\text{-}high}$ in the hair matrix. It should be emphasized that Mb/Mc display the same patterns as surrounding cells in terms of expression of E- and P-cadherin, suggesting that mimicry of the cadherin expression pattern is essential for stable settlement of Mb/Mc in different tissues.

It is difficult to determine whether the striking concordance in the cadherin expression pattern between Mb/Mc and surrounding cells is intrinsic or extrinsic. Obviously, it is not related to the differentiation stage of the Mc lineage, because the Mc remaining in the dermis of hairless regions are E-cad$^-$P-cad$^-$ throughout the differentiation process, including immature bipolar Mb and fully pigmented dendritic Mc. We recently showed that the forced expression of SLF in the basal layer of the epidermis in transgenic mice allows epidermal Mb/Mc to survive even in the postnatal life (Kunisada *et al.*, 1998b). Taking advantage of the transgenic mice, we showed that epidermal Mb/Mc also maintain an epidermal type surface phenotype (E-cad^{high}P-cad^{low}) during the postnatal life, indicating that this pattern correlates closely with the localization of Mb/Mc. We thus prefer the possibility that the expression patterns of E- and P-cadherins in Mb/Mc are regulated extrinsically by microenvironmental cues.

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